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Award Number: DAMD17-99-1-9249

TITLE: Functional Sites of the erbB-2 Receptor and Its Activator Heregulin

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REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual Summary (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
Functional Sites of the *erbB-2* Receptor and Its Activator Heregulin

5. FUNDING NUMBERS
DAMD17-99-1-9249

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Aberrant expression of the *erbB-2* (HER-2/neu) receptor occurs in up to 30% of human breast cancers and correlates with aggressive disease and poor prognosis. The growth factor heregulin (HRG) binds to *erbB-3* or *erbB-4*, promoting heterodimerization with *erbB-2*, inducing autophosphorylation and activation of *erbB-2* signaling. It is generally accepted that HRG and *erbB-2* do not interact directly. Depending on its concentration HRG can either inhibit or stimulate cell proliferation in cell lines that overexpress *erbB-2*. This suggests some type of direct interaction between HRG and *erbB-2*. Solution structure of HRG and other data support the existence of a low affinity-binding site within the EGF-like domain of HRG. **The goal of the proposed experiments is to define the predicted sites of interaction between HRG and the *erbB-2* receptor through generation of HRG and *erbB-2* deletion mutants.**

During the first year of funding, I maintained the timeline outlined in the statement of work: a) generated deletions mutants of *ErbB-2* lacking the putative heregulin binding site or site important for receptor heterodimerization and b) initiated the construction of heregulin mutants. The main result of my work was generating 8 deletions of the *ErbB-2* extracellular domain. These mutants will be used in the experiments determining the mechanism of interaction between *ErbB-2* and heregulin.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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5. INTRODUCTION

The epidermal growth factor (EGF) receptor family comprises four transmembrane tyrosine kinases (EGFR, *erbB-2*, *erbB-3* [kinase defective], *erbB-4*) that are involved in the genesis and progression of a variety of human carcinomas. Amplification and overexpression of the *erbB-2* (HER-2/neu) receptor tyrosine kinase receptor, occurs in up to 30% of human breast cancer and has been shown to correlate with aggressive disease and poor prognosis. Despite the involvement of *erbB-2* in tumor development, its exact biological functions have yet to be elucidated. Heregulin (HRG, NDF) is a 45 kDa growth factor that is expressed in about 30% of breast carcinomas and is associated with invasion and metastasis. HRG was initially isolated as a specific ligand for *erbB-2*, however, it is generally accepted that HRG does not bind directly to *erbB-2*. HRG binds to *erbB-3* and *erbB-4* with high affinity. HRG binding to *erbB-3* or *erbB-4* promotes heterodimerization with *erbB-2*, inducing autophosphorylation and activation of *erbB-2* signaling.

It was recently found that HRG does have an extremely low affinity for *erbB-2*. Solution structure of HRG indicates an EGF-like domain of HRG with two clusters of amino acids apparently involved in receptor binding: a conserved high-affinity binding domain of 35-40 amino acid residues located in the N-terminal portion and a region of non-conserved amino acids comprising a low affinity binding domain in the C-terminal portion. HRG-neutralizing antibodies prepared in Dr. Lupu's laboratory directed against the C-terminus (α1) and the N-terminus (α3) blocked HRG-induced growth effects. However, the antibodies failed to block *erbB-2* receptor phosphorylation when used separately; blockage of receptor phosphorylation was observed only when the antibodies were used in combination. These observations prompted to hypothesize that the C-terminus constitutes a site for low affinity binding between HRG and *erbB-2*.

The extracellular region of *erb-B* receptors has been organized into a four-domain model, in which subdomain III contributes most of the determinants involved in ligand binding and signal transduction. To determine the putative site involved in the interactions between *ErbB-2* and growth factors, a number of synthetic peptides with sequences homologous to specific *erbB-2* regions (termed the RL series) were generated in Dr. Lupu's laboratory. It was shown that the peptide RL2, which was derived from a sequence in the *erbB-2* extracellular domain, was capable of specifically blocking HRG-induction of *erbB-2* tyrosine phosphorylation. Due to this fact, it is possible that this region (in the proximity of the RL2 peptide) constitutes a critical region of the *erbB-2* receptor responsible for HRG induction of *erbB-2* heterodimerization and activation.

The goal of this project is to explore the therapeutic potential of HRG for use against breast cancers, which overexpress *erbB-2*, through the:

a) **Generation of HRG mutants that will exclusively bind to *erbB-2* and**
b) **Identification of the *erbB-2* functional site (site responsible for receptor heterodimerization).**

Identification of the specific site of interaction between HRG and *erbB-2* as well as identification of the *erbB-2* site responsible for HRG induction of receptor heterodimerization will enhance the necessity of developing HRG targeted agents including, HRG antagonist and/or HRG-targeted vehicles that could improve the delivery of chemotherapeutic agents.

6. BODY

The goal of the research in this proposal is to extend the ongoing studies. The following experiments are designed to shed light on the biological and molecular mechanism by which heregulin can induce the activation the *ErbB-2* signaling pathway of epithelial cells that leads to the aggressive forms of tumors. The original technical objectives were as follows:

- **Task 1: To generate deletion mutants of the *erbB-2* extracellular domain in order to determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation and/or its heterodimerization with other members of the *erbB* receptor family.** These studies will be accomplished by generating the series of *erbB-2* deletion mutants and subsequent transfection of the obtained constructs in the retroviral vector into the Ba/F3 cell pro-B-lymphocyte cell line. Following the transfection the cells will be tested in order to determine their ability for growth and proliferation in the presence or absence of the wild type HRG.

- **Task 2: To generate HRG deletion mutants (Δ-HRG) and determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling.** I will generate two sets of heregulinβ mutants. One to the N-terminal domain, that contains the low affinity binding site involved in the direct type of interaction with *erbB-2*; second to the EGF-like domain binding to the *erbB-3* and stimulating its dimerization with other members of receptor tyrosine kinases family. These studies will be accomplished by partial deletions within critical domains and subsequent point mutations to determine which of amino acids are critical for interactions with the receptor. The Ba/F3 cell pro-B-lymphocyte cell line transfected with *erbB* receptors will treated with wild type and mutant HRG in order to test if the *erbB-2* signal transduction pathway is active.

STATEMENT OF WORK:

Task 1: To generate deletion mutants of the *erbB-2* extracellular domain in order to determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation and/or its heterodimerization with other members of the *erbB* receptor family.

ACCOMPLISHED OBJECTIVES FROM INITIAL TASK 1 AND 2:

A) ***Development of erbB-2 mutants lacking the putative HRG binding site or the site responsible for receptor heterodimerization, corresponding to the RL2 region.*** (Months 1-10)

During the course of the studies, I decided to construct eight different deletion mutants within subdomain III of the extracellular domain of *erbB-2* using the polymerase chain reaction methodology (PCR). I took this approach because of the following advantages:

- It has been proven efficient and reliable.
- It does not require the generation of specific enzymatic restriction sites for each one of the mutants.
- It enables to create the deletion mutants without changing their initial open reading frame (ORF), which is important to maintain the protein structure.

Generation of the *erbB-2* mutants: To generate the *erbB-2* deletion mutants, I initially used the full-length *erbB-2* cDNA. To simplify the construction of the mutants I selected a unique 1826 bp fragment, between to unique restriction enzymes: *Sse8387I* and *BspEI.* Using specific DNA primers, generated according to the appropriate *erbB-2* sequences for each one of the deletions, eight different mutants were generated using a two steps PCR procedure. In the first PCR reaction, each one of the regions of the *erbB-2* mutant (upstream and downstream) was generated, as can be seen in Figure 1.

Generated fragments that contained complementary ends in order to anneal to each other and derive the final expected product during the amplification of the second PCR reaction as demonstrated in Figure 2. As can be seen, we were able to successfully generate all of the predicted deletion mutants.

The next step in the process of completing this task was the introduction of modified *erbB-2* fragments into the full-length sequence of the parental *erbB-2* gene. This portion of the task has becoming more complicated, than what we originally expected. This complication was due to the fact that one of the selected enzymes to insert the mutated fragment into the full-length sequence was fund to be unexpectedly in the vector as well. Therefore, we opted to use another vector at first and then after completing the annealing of all the fragments to transfect the deleted *erbB-2* mutants into the expression vector as originally planed. We are currently in the process of completing this task.

Task 2: **To generate HRG deletion mutants (Δ-HRG) and determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling.** Months 1-8: To generate HRG deletion mutants.

After extensive current literature searches and sequence analysis of the heregulin gene, I decided to generate initially four HRG mutants. I have currently designed the initial sequences to obtain the initial PCR primers to generate the HRG mutants by site directed mutagenesis. The oligos (PCR primers) that I have designed were designed to match to nine amino acids, within the heregulin EGF-like domain. We predicted that these amino acids are involved in the interactions with *erbB* receptors.

TASKS REMAINING TO BE PERFORMED FROM INITIAL TASKS 1 AND 2:

Task 1: **To develop the cell lines containing mutated *erbB-2* receptors in order to determine the specific functional sites on *erbB-2*.** Months 10-18: Transfection of the *erbB-2* mutants into Ba/F3 cells and specific clones isolation.

Task 2: **To determine if the C-terminal domain of HRG is a low affinity binging site that is specific and unique for *erbB-2*.** Months 8-14: Expression of Δ-HRG recombinant protein.

TASKS REMINING TO BE PERFORMED DURING THE NEXT YEAR FROM THE INITIAL STATEMENT OF WORK

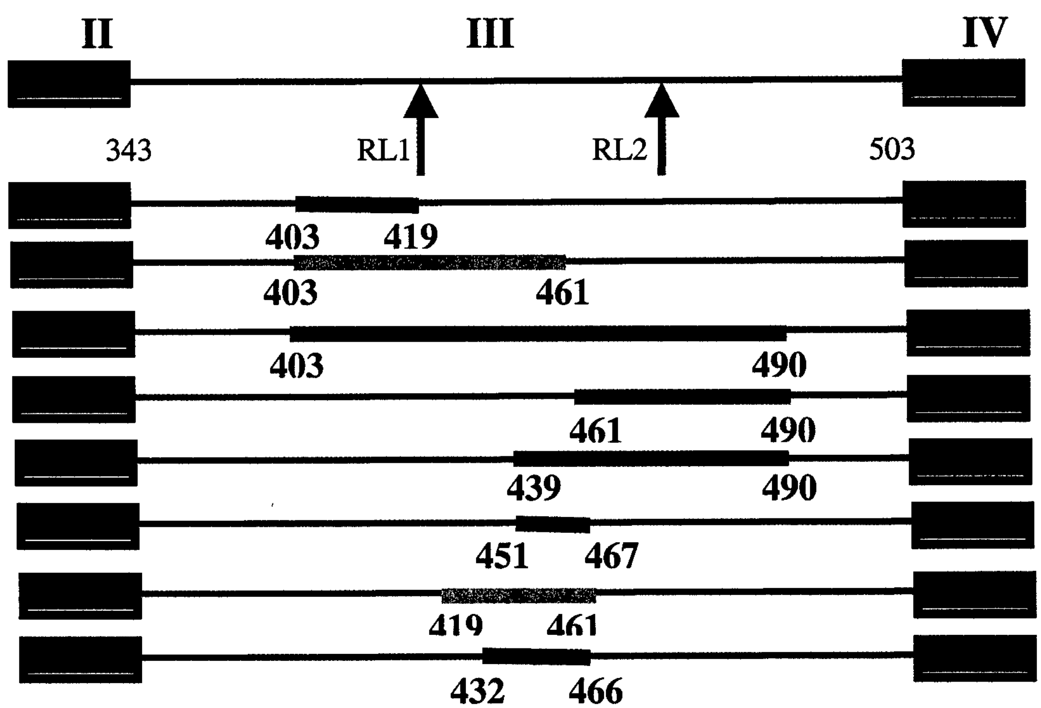
Task 1: **To determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation.** Months 18-24: To perform phosphorylation and immunoprecipitation assays.

Task 2: **To determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling.** Months 14-16: To perform sequence analysis in order to check if the introduced mutations did not have changed the open reading frame. Months 16-24: To perform phosphorylation assays to test the HRG mutants for their ability to induce tyrosine phosphorylation of the *erbB* receptors.

7. KEY RESEARCH ACCOMPLISHMENTS

- I have successfully generated eight *erbB-2* deletion mutants in the predicted sequence located at the predicted *erbB-2* functional site. All of the mutants are deletions of sequences at the subdomain III of the *erbB-2* extracellular domain. The mutants maintained the open reading frame. Here is the summary of the deletion mutants.

    1. Deletion 1 - 403- 419 aa (control)
    2. Deletion 2 - 403- 461 aa
    3. Deletion 3 - 403- 490 aa
    4. Deletion 4 - 461- 490 aa
    5. Deletion 5 - 439- 490 aa
    6. Deletion 6 - 451- 467 aa
    7. Deletion 7 - 419- 461 aa
    8. Deletion 8 - 432- 466 aa



- I have performed an extensive literature search and analyze which are the most important areas of the HRG gene to be a functional site of the HRG interaction with the erbB- receptors. I have then designed the optimal oligo-nucleotide sequences to generate these mutants by site directed mutagenesis of the heregulinβ2 isoform.

8. REPORTABLE OUTCOMES

- Generation of the *erbB-2* deletion mutants at the putative functional site.

9. CONCLUSIONS

The original goal of this proposal was generate DNA deletion constructs of the erbB-2 and Heregulin sequences for subsequent functional studies and to determine their biological significance in breast cancer.

- The generation of the putative deletion mutants has unexpectedly taking longer time because of unpredicted difficulties in the experimental design. We have successfully overcome those difficulties and finally generated eight erbB-2 deletion mutants. These mutants will be shortly transfected into BaF3 cells and their biological function will be determined.
- We are determined the putative functional sites of the HRG sequences at the EGF-like domain.

These experiments will enable the further studies on defining the possible direct interactions between *erbB-2* and HRG. Our future work is aimed at defining the functional sites involved in cross-talk between these proteins. Better understanding of this process can provide new strategies to stop or slow down breast cancer progression.

10. REFERENCES

Not Applicable

11. APPENDICES

Appendix I: Figures and Figure legends

Figure 1. PCR products for deletion mutations of *erbB-2* obtained after first PCR reactions. Numbers correspond to the subsequent mutants; U is for the fragments upstream of deletions, D is for the fragments downstream of deletions; M 100 is 100bp DNA ladder, M 1 is 1kB DNA ladder.

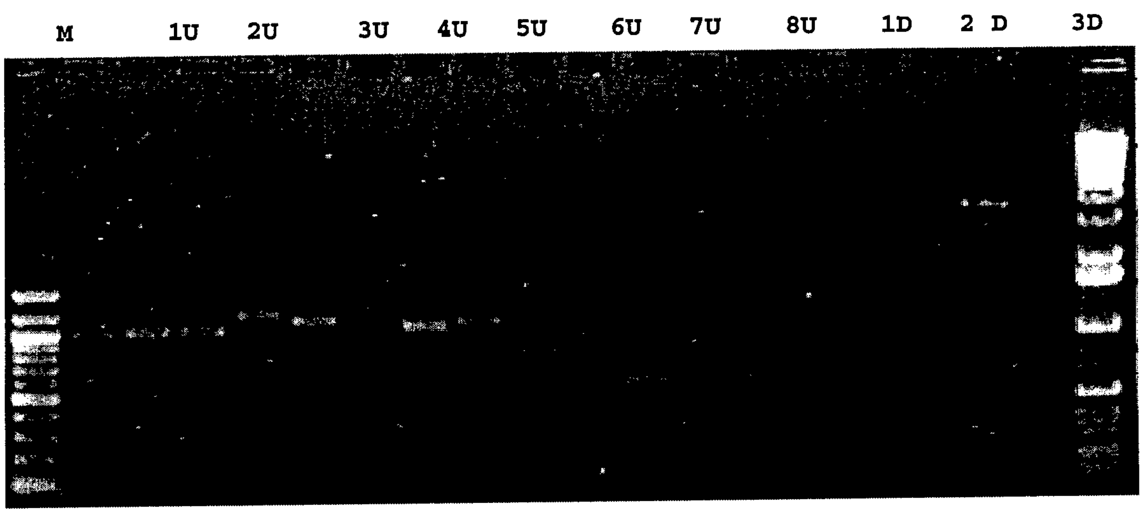


Figure 2: PCR products for deletion mutations of *erbB-2* obtained after second PCR reactions. Numbers correspond to the designed deletion mutants respectively, WT is wild type fragment, M is molecular weight, DNA ladder.